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Published*With international search report.***(54) Title:** DETECTION OF NITRIC OXIDE USING ELECTRON SPIN RESONANCE**(57) Abstract**

A method of detecting nitric oxide in a sample utilizing electron paramagnetic resonance spectroscopy and a spin-labeled material such as fusinite to detect changes in the linewidth of the electron paramagnetic resonance signal of the spin-labeled material in the presence of nitric oxide which can be correlated to the quantity of nitric oxide in the sample.

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DETECTION OF NITRIC OXIDE
USING ELECTRON SPIN RESONANCE

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FIELD OF THE INVENTION

This invention relates to a nondestructive method of detecting nitric oxide. In particular, this invention enables the measurement of nitric oxide specifically and quantitatively in aqueous and nonaqueous solutions of 10 biological media, both in vitro and in vivo, and chemical media.

BACKGROUND OF THE INVENTION

Nitric oxide (NO) is a key bioregulatory molecule 15 that plays critical roles in the regulation of various biological processes, including the normal physiological control of blood pressure, macrophage-induced cytostasis and cytotoxicity, inhibition of platelet aggregation, and neurotransmission (Moncada et al., *Pharmacological 20 Reviews* 43(2): 109-142. 1991.). Many tissues in the body endogenously release NO in different amounts (Marletta, *Chem. Res. in Toxicology* 1(5): 249-257. 1988.; *Biochemistry* 27: 8706-8711. 1988.) but the actual amounts released are very difficult to quantify. In addition, 25 many diseases, such as endotoxic shock, ischemia reperfusion injury, genetic mutations, which include deamination-related genetic diseases (Wink et al., *Science* 254: 1001-1003. 1991.) like deamination of cytosine to thymine, cancer, male impotence, and 30 atherosclerosis have been suggested to be caused by defects in the production and/or regulation of NO (Moncada et al.; Masini et al., *Agents and Action* 33: 53-56. 1991.). Also, drugs, including xenobiotics, can 35 be metabolized to give NO either as the effector molecule or as a harmful metabolite (Feeleisch, *J. Cardiovasc. Pharmacol.* 17: S25-S33. 1991.; Ignarro et al., *Biochem. Biophys. Res. Comm.* 94: 93-100. 1980.; Servent et al., *Biochem. Biophys. Res. Comm.* 163: 1210-1216. 1989.; and Haussmann et al., In: Relevance of N-Nitroso Compounds to

Human Cancer. Exposures and Mechanisms. Bartsch, O'Neill and Schulte-Hermann, eds. IARC Sci. Pubs. 84: 109-112. 1987.).

5 The importance of the bioregulation effected by NO is further evidenced by the recent rash of pharmaceutical companies designing drugs around NO. It is hoped that drugs can be developed to control blood pressure, prevent atherosclerosis, treat migraine headaches and impotence, prevent deaths from septic shock, and help protect brain 10 cells threatened by degenerative diseases and strokes.

Accordingly, the ability to nondestructively measure NO concentrations specifically and quantitatively in aqueous and nonaqueous solutions of biological media, both in vitro and in vivo, and chemical media would be 15 highly advantageous. The ability to measure the concentration of NO by a nondestructive method, e.g., in a manner which does not consume NO is an important requirement for further investigation of the mode of action of NO as a key bioregulatory molecule and for the 20 development of therapeutic applications of NO-releasing compounds. Several techniques have been employed to determine the concentration of NO in aqueous solution.

One method employs an automated system that analyzes nitrate by reduction with a high-pressure cadmium column 25 to determine amounts of nitrate and/or nitrite in urine, saliva, deproteinized plasma, gastric juice, and milk samples (Green et al., Analytical Biochemistry 126: 131-138. 1982.). The lower limit of detection of the method is said to be 1.0 nmol NO_3^- or NO_2^- /ml. The system 30 reportedly allows quantitative reduction of nitrate and automatically eliminates interference from other compounds normally present in biological fluids. Most samples may be prepared by simple dilution with distilled water, and 30 samples reportedly may be analyzed in an 35 hour. The disadvantage of such a technique in measuring NO is that it does so indirectly, by measuring NO byproducts, which also can be generated from other

sources. Accordingly, such a method is not very accurate in determining NO concentration.

Another method quantitatively analyzes nitrite, an oxidation product of NO, in human plasma to determine NO concentration (Wennmalm et al., *Analyt. Biochem.* 187: 359-363. 1990.). Dithionite is used to treat the samples of human plasma to convert nitrite to nitric oxide, with the treated samples being passed over bovine hemoglobin columns. NO is allowed to bind the hemoglobin in columns of bovine hemoglobin covalently bound to agarose. An excess of dithionite is used to ensure that the hemoglobin is reduced to a ferrous, nonoxygenated state. The NO bound to the hemoglobin forms a complex on the column, and the column is then subjected to electron paramagnetic resonance spectroscopy, i.e., the column is subjected to a magnetic field and microwave radiation to obtain a characteristic electron paramagnetic resonance spectrum. This method suffers from the same disadvantages as the previously described method. NO concentration is determined indirectly, through the measurement of nitrite. Also, the NO is modified by binding to hemoglobin covalently bound to agarose.

Other methods employed to quantitate NO include chemiluminescence, mass spectroscopy (Bazyliński et al., *Inorg. Chem.* 24: 4285-4288. 1985.), and ultraviolet-visible light spectral changes. In one procedure utilizing chemiluminescence, NO has been quantified by the chemiluminescence resulting from the product of NO and ozone (Palmer et al., *Nature* 327: 524-526. 1987.; Maragos et al., *J. Med. Chem.* 34: 3242-3247. 1991.). This method also involves modification of NO, in this case by reaction with ozone. In a procedure employing ultraviolet and visible light, spectral changes have been monitored for the conversion of oxyhemoglobin to methemoglobin by NO as an indication of NO concentration (Haussmann et al.). NO is modified in this method by reaction with oxyhemoglobin.

Accordingly, neither one of these methods enables the measurement of NO directly.

Solution methods have been also used to measure NO but seem to lack specificity for NO or reliable quantitation. The use of 3,5-dibromo-4-nitrosobenzene sulphonate (DBNBS) as a spin trap in an electron spin resonance technique to detect NO in a biological system has been reported (Arroyo et al., *Biophys. Res. Comm.* 170: 1177-1183. 1990.).

This method, consequently, involves reaction of NO with modified spin traps. Subsequently, it was demonstrated that the obtained signal may result from simple oxidation of the spin trap, which raises the issue of how specific the spin trap is for NO (Wink et al., *Radiat. Phys. Chem.* 38: 467-472. 1991.).

The use of Fe^{2+} (dithiolate) to trap NO as the nitrosyl also has been used in a spin resonance technique (Mulsch et al., *FEBS Letter* 294: 252-256. 1991.); however, this technique is not suitable for quantitation due to a lack of biological stability, i.e., the resulting nitrosyl has a half-life of only about 30 seconds in biological systems. Further, it is evident that this method involves the modification of NO by formation of a complex with iron. The iron complex is metabolized, i.e., destroyed, during the process. Also, this method suffers from nitrite interference.

More recently, a modified oxygen electrode has been used to detect NO (Shibuki et al., *Neuroscience Res.* 9: 69-76. 1990.; *Nature* 349: 326-328. 1991.). The electrochemical microprobe was developed to detect the release of NO in brain tissue. The output current of the probe was found to correlate linearly with the concentration of NO at the tip. The sensitivity of the probe was reportedly between 3.5 and 106 pA/ μ M change in NO concentration.

However, the validity of this technique has been questioned due to the small current that has been observed (<0.5 pA) and the lack of use of standards at submicromolar concentrations of NO. In

addition, the technique measures NO by its oxidation to nitrites and those who developed the modified oxygen electrode claim that NO is spontaneously released from sodium nitroprusside and that the release is accurately measured by the electrode. This contradicts what has been shown previously by others, i.e., that sodium nitroprusside does not spontaneously release NO in buffer (Kruszyna et al., *Toxicol. Appl. Pharmacol.* 91: 429-438. 1987.; Wilcox et al., *Chem. Res. Toxicol.* 3: 71-76. 1990.), which raises the issue of specificity of this method.

There is a need, therefore, for a nondestructive method, e.g., nonconsuming method, of detecting NO and of measuring NO concentration in a specific, quantitative, and reproducible manner. The methods described above result in the destruction of NO, necessitate the modification of NO, e.g., its consumption through, for example, modification of NO, in order to measure NO concentration, are invasive procedures, require extrapolation to an earlier event in order to determine the concentration of NO. The present invention not only provides a method that enables the detection and measurement of NO concentration specifically, quantitatively, and reproducibly in a nondestructive manner, but it enables the ability to nondestructively assay NO concentration over time. The other methods do not allow such a real time assay to both readily and reliably determine NO concentration.

The present inventive method employs electron spin resonance. Electron spin resonance (ESR) has been used to measure oxygen concentration. Recently, fusinite, a naturally occurring polymeric solid component of many coals, has been shown to give an ESR signal upon L-band irradiation. Rich in unpaired electrons, fusinite has been shown to generate a single electron paramagnetic resonance (EPR) signal that broadens in the presence of oxygen (Clarkson et al., *Fuel* 69: 1405-1411. 1990.). The

line broadens as a function of oxygen concentration in a reproducible fashion.

When properly isolated from whole coal, purified, and ground to a fine powder ($d \leq 5 \mu\text{m}$), fusinite provides a useful probe for EPR measurement of oxygen concentration. The utility of fusinite in the EPR measurement of oxygen concentration in vivo has been demonstrated in cells and animals (Swartz et al., Magnetic Resonance Medicine 20: 333-339. 1991.). The advantages of fusinite in such a technique are very low toxicity, excellent chemical stability, and sensitivity to concentrations as low as $0.1 \mu\text{M}$. In fact, fusinite can be easily placed inside a cell and can be retained within an organism for as long as a year without loss of signal or development of toxic side effects.

It was not believed that fusinite and EPR also could be used to measure NO concentration, particularly in a reliably specific and quantitative manner. Although oxygen and NO are both paramagnetic gases, the two gases differ in at least one very significant respect. Oxygen is not a radical species, whereas NO is a radical species. Given that radicals are known to be short-lived and highly reactive and that fusinite is a coal derivative that has many potential reaction sites, one who is skilled in the art would have predicted that a coal derivative, such as fusinite, and EPR could not be used to measure NO concentration. One would have expected that the NO radicals would react with metals, sulfur compounds, and possibly other radical species in the coal derivative. Such reactions include radical couplings, which would result in the formation of diamagnetic species, thereby resulting in the loss of the EPR signal from the fusinite. The loss of the EPR signal would prevent the determination of the concentration of NO in a sample.

Surprisingly, it has been found that fusinite does not chemically react with NO, and, therefore, it is

possible to obtain an EPR signal for NO using fusinite and EPR spectroscopy, which enables the measurement of the EPR linewidth, its comparison with EPR linewidths for known signals, and the subsequent determination of NO concentration. The ability to measure successfully NO concentration using EPR and a coal derivative, such as fusinite, as provided by the present invention is an unexpected result, which was neither taught nor suggested in the art. In addition, the present inventive method overcomes the deficiencies of the methods currently being used to measure NO concentration by providing a nondestructive, e.g., nonconsuming, method that enables the measurement of NO concentration specifically, quantitatively, and reproducibly, in aqueous and nonaqueous solutions of biological media, both in vitro and in vivo, and chemical media. It also enables real time assay of NO concentration.

SUMMARY OF THE INVENTION

The present invention provides a nondestructive method, which utilizes a spin-labeled material, particularly a derivative of coal, such as fusinite, and EPR, for the detection of nitric oxide. The present inventive method of detecting nitric oxide comprises contacting a sample of unknown nitric oxide concentration with a spin-labeled material, subjecting the unknown sample and spin-labeled material to EPR spectroscopy under hypoxic conditions to obtain an EPR signal having a linewidth, and comparing the EPR linewidth for the unknown sample and spin-labeled material to the EPR linewidth of an EPR signal for the spin-labeled material in the presence of a sample or samples of known nitric oxide concentration in order to detect the presence, and determine the concentration, of nitric oxide in the unknown sample.

The method enables the measurement of NO specifically and quantitatively in aqueous and nonaqueous

solutions of biological, both in vitro and in vivo, and chemical media in a reproducible manner. The present invention also enables the real time assay of NO concentration.

5 The present invention further provides a means of monitoring NO production or inhibition by drugs, both in vitro and in vivo, in the design of drugs for the treatment of diseases related to defects in NO regulation and/or production as well as a means of detecting and
10 quantifying defects in NO regulation and/or production, both in vitro and in vivo, which result from disease, injury, and mutation.

The present invention additionally provides a means of monitoring pollution of which NO is a component.

15 These and other objects and advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph of EPR linewidth (milligauss) versus NO concentration (μM) that shows the variation in the EPR linewidth for fusinite powder suspended in a 0.1 M phosphate buffered saline at pH 7.4 and 35°C subjected
25 to varying pressures of NO. Measurements were made in an EPR tube at X-band (8.9-9.6 GHz).

Figure 2 is a graph of NO concentration (μM) versus time (seconds) that shows the variation in NO concentration with time as measured using EPR and fusinite for the NO-releasing compound DEANO in 0.1 M phosphate buffered saline at pH 7.4 and 35°C. The dashed line represents the kinetics for this reaction at 37°C as predicted from the data of Maragos et al. (J. of Medicinal Chem. 34: 3242-3247. 1991.), who followed the
30 reaction using an optical absorbance technique and calculated an apparent rate constant $k = 5.4 \times 10^{-3}/\text{sec}$ for this system.

Figure 3 is a graph of NO concentration (μM) versus time (seconds) that shows the variation in NO concentration with time as measured using EPR and fusinite for the NO-releasing compound SPERNO in 0.1 M phosphate buffered saline at pH 7.4 and 35°C. The dashed line represents the kinetics for this reaction at 37°C as predicted from the data of Maragos et al. (J. of Medicinal Chem. 34: 3242-3247. 1991.), who followed the reaction using an optical absorbance technique and calculated an apparent rate constant $k = 3.0 \times 10^{-4}/\text{sec}$ for this system.

Figure 4 is a graph of signal amplitude (A.U.) versus magnetic field (Gauss) that shows the immediate narrowing of the EPR linewidth for fusinite upon injection of pure oxygen gas into the reaction cell following the conclusion of the DEANO reaction, i.e., when no further change in the linewidth was observed.

Figure 5 is a graph of EPR linewidth (Gauss) versus time (minutes) that shows the initial decrease and subsequent increase in the EPR linewidth for fusinite in the presence of NO over time as measured in Chinese hamster ovary cells in vitro.

Figure 6 is a graph of EPR linewidth (milligauss) versus NO concentration (μM) that shows the variation in the EPR linewidth for ^{15}N -perdeutero TEMPONE suspended in 0.1 mM phosphate buffered saline at pH 7.4 and 35°C subjected to varying pressures of NO. Measurements were made in an EPR tube at X-band (8.9-9.6 GHz).

30 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated on the discovery that EPR and a coal derivative, such as fusinite, may be used to detect nondestructively and measure specifically and quantitatively the concentration of NO in aqueous and nonaqueous solutions of biological media, both in vitro and in vivo, and chemical media, in a reproducible manner with a limit of detection $\leq 0.2 \mu\text{M}$. The present

inventive method does not result in the consumption of NO, e.g., through modification of NO, and, therefore, is a technique, thereby rendering the present inventive method quite suitable for in vivo, as well as in vitro, 5 detection and measurement of NO.

The present inventive method utilizes EPR spectroscopy, which is a technique that is well-known to those who are skilled in the art. It was found that the EPR linewidth for fusinite is sensitive to the presence 10 of NO and that the variation in the EPR linewidth for fusinite can be used to determine the concentration of NO in a sample under test.

The present inventive method of detecting NO specifically comprises contacting a sample of unknown NO 15 concentration with a spin-labeled material, subjecting the unknown sample and spin-labeled material to EPR spectroscopy under hypoxic conditions to obtain an EPR signal having a linewidth, and comparing the EPR linewidth for the unknown sample and spin-labeled 20 material to the EPR linewidth of an EPR signal of the spin-labeled material in the presence of a sample or samples of known NO concentration in order to detect the presence, and determine the concentration, of NO in the unknown sample. The method of the present invention can 25 be used to merely detect the presence of NO in an unknown sample, for example by comparison of the observed EPR linewidth to the EPR linewidth for the spin-labeled material in the absence of NO, or to determine the concentration of NO in the unknown sample, for example by 30 comparison of the EPR linewidth to the EPR linewidths for the spin-labeled material in the presence of different NO concentrations.

It is believed that other spin-labeled materials, which do not chemically react with NO, may also be used 35 in the context of the present invention. Such other spin-labeled materials are coal derivatives that have characteristic features in common with fusinite, burnt or

partially combusted cellulose, irradiated gas-permeable plastics, and spin-labeled molecules, such as TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl), DOXYL (4,4-dimethyloxazolidine-N-oxyl), and PROXYL (2,2,5,5-tetramethylpyrrolidine-N-oxyl) (all available from Sigma, St. Louis, MO), and spin-labeled molecules that have characteristic features in common with the spin-labeled molecule(s) of fusinite. Moreover, the spin-labeled molecule(s) of fusinite could be isolated for use in the present inventive method.

Fusinite, TEMPO, and derivatives thereof are most preferably utilized as the spin-labeled materials in the context of the present invention. In particular, spin-labeled compounds which have been modified by use of isotopes with lower spin numbers, e.g., the use of ¹⁵N for ¹⁴N and deuteration, can lead to greater sensitivity of the present inventive method. For example, ¹⁵N-perdeutero TEMPONE (¹⁵N-perdeutero 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl or 4-OXO-TEMPO) (Sigma, St. Louis, MO) results in a greater sensitivity of EPR spectroscopy as applied in the context of the present invention as compared to the use of TEMPO. This greater sensitivity is a result of the use of ¹⁵N leading to a two line, rather than three line, EPR pattern and deuteration leading to a narrower EPR linewidth in the absence of a paramagnetic species.

Since EPR in combination with fusinite has been demonstrated to have utility in the detection and measurement of oxygen, it is important that the use of EPR in combination with fusinite in the detection and measurement of NO be carried out under hypoxic conditions so that the variation in the EPR linewidth for fusinite accurately reflects the concentration of NO in the sample under test. However, it will be appreciated by one who is skilled in the art that the chemical reactivity of NO and O₂ may offer the opportunity to follow the titration

of NO by physiological oxygen levels in cells and tissues.

The method of the present invention may be used to detect the presence of NO or measure the concentration of NO in a wide variety of samples. The method may be used to test aqueous and nonaqueous solutions of biological and chemical media. The testing of biological media may be carried out in vitro or in vivo. In fact, the present inventive method may be used to detect and/or measure the presence of NO in a biological fluid, such as blood, urine, or saliva, cells, tissues, or even an intact organism. The use of the present inventive method to measure the presence of NO in an intact organism would enable imaging of tumor cells, which are known to produce large quantities of NO. Of course, the application of the present inventive method to the testing of intact cells or tissues in vivo or an intact living organism would require utilization of a modified ESR spectrometer, for example, in which the magnets that generate the magnetic field are positioned in an upright manner so as to form a tabletop for positioning of the cells, tissues, or intact organism for testing. Since the present inventive method provides for the rapid determination of NO concentration, as well as being nondestructive, e.g., nonconsuming of NO, it is well suited for use in real time procedures, particularly on living organisms such as humans in need of either constant monitoring or emergency medical diagnosis and treatment.

In addition, the temperature and band of irradiation at which the testing is done may necessarily vary with the sample under test. Generally, however, testing in the temperature range from about 4°C to about 90°C, preferably from about 20°C to about 40°C, and more preferably from about 20°C to about 37°C, and irradiation from about L-band (1-2 GHz) to about X-band (8.9-9.6 GHz) is preferred. Higher frequency irradiation may be used. However, penetration decreases and heat generation

increases with an increase in frequency. For example, at Q-band (34-36 GHz), it is believed that an ESR signal still can be obtained but that the sensitivity decreases significantly. Also, the ability to penetrate fusinite 5 also decreases. Accordingly, irradiation at such high frequencies requires the use of finer particles of fusinite or the like and also necessitates cooling of the material under test, since irradiation at such high frequencies generates much heat. L-band is preferred for 10 intact organisms in order to avoid tissue breakdown and cell death.

Prior to operation, the system should be calibrated, such as described in Example 2 of the present specification. The EPR linewidth, ΔB_{pp} , obtained as a 15 function of paramagnetic gas pressure in accordance with this method results in a very stable calibration that allows for the measurement of unknown gas pressures from linewidth measurements (Swartz et al., Magnetic Resonance Medicine 20: 333-339. 1991.). It has been further 20 established, using a Clark electrode, that gas phase calibrations are accurate even when the probe material, fusinite in this case, is suspended in an aqueous solution and the relationship 1 mm Hg = 1.3 μ M is used to convert gas pressure to solution concentration.

25 Measurements at 1 GHz and 250 MHz have proven that calibrations at X-band (8.9-9.6 GHz) frequencies also retain their validity when measurements are made at lower microwave frequencies and lower magnetic fields. Such calibrations, wherein a standard curve is obtained, may 30 be also generated in an intact organism. It will be appreciated by one who is skilled in the art that other methods of calibrating the EPR system may be used.

Once the EPR system is calibrated, the variation in or the broadening of the EPR linewidth for fusinite or 35 the like, such as a spin-labeled molecule, such as TEMPO or a spin-labeled molecule isolated from fusinite, in the presence of NO may be measured. Once the variation in

the EPR linewidth for the spin-labeled material has been measured, the concentration of NO is determined by extrapolation from the standard curve generated during calibration of the system.

5 The present inventive method is also expected to have utility in monitoring pollution of which NO is a component, in monitoring NO production or inhibition by drugs, both in vitro and in vivo, in the design of drugs for the treatment of diseases related to defects in NO 10 regulation and/or production, and in detecting and quantifying defects in NO regulation and/or production, both in vitro and in vivo, which result from disease, 15 injury, and mutation. For example, in an emergency room, septic shock may be distinguished from trauma by high and low NO concentration, respectively. One who is skilled 20 in the art will appreciate that other applications of the present inventive method are possible.

 The following examples serve to further illustrate the present invention and are not intended to limit the scope of the invention.

EXAMPLE 1

 This example describes the preparation of fusinite. Large, pure fusinite lenses were hand-selected from 25 an Illinois No. 5 coal. The hand-selected lenses were washed in hot, dilute hydrochloric acid and rinsed in triply distilled water. The washing and rinsing of the lenses was repeated twice more. The resulting powder, upon L-band irradiation, gave a single EPR resonance 30 signal, free from inorganic paramagnetic ion contamination.

 Samples of the resulting powder were air-dried and ground to pass through sieves of mesh. The finest mesh allowed particles with a diameter of less than 10 35 micrometers to pass through. The resulting fusinite powders were then stored in air until needed.

EXAMPLE 2

This example describes the calibration of the EPR linewidth for the fusinite powder as a function of NO concentration.

5 The EPR linewidth for the fusinite powder was calibrated as a function of NO concentration utilizing a dynamic vacuum system that allowed a small quantity of fusinite to be placed in an X-band (8.9-9.6 GHz) EPR cavity. Quantities of NO gas (ACS reagent grade, 10 Matheson, Rutherford, NJ) were admitted to the system and the equilibrium gas pressure was measured using a Varian Thermatron thermocouple gauge calibrated against a mercury McLeod gauge. EPR spectra were obtained for fusinite at various equilibrium NO pressures using an EPR 15 spectrometer (Bruker Co., MA).

Figure 1 shows the variation in EPR linewidth for a fusinite powder subjected to varying concentrations of NO. The nonlinear behavior of ΔB_{pp} as a function of NO is similar to that observed for oxygen (Swartz et al., 20 Magnetic Resonance Medicine 20: 333-339. 1991.). Other paramagnetic species in solution, such as nitroxide radicals and Fe^{+3} ions, have been reported to have no effect on the linewidth of fusinite, presumably due to size exclusion by the fusinite pore system and the rather 25 hydrophobic character of the interior surfaces. Furthermore, no linewidth effects have been reported in the presence of strong oxidizing gases, such as F_2 , strong acids, or bases. This suggests that only paramagnetic gases can alter the linewidth of fusinite in 30 aqueous and gas/solid systems.

EXAMPLE 3

This example demonstrates the use of fusinite and EPR in the measurement of NO concentration in solutions 35 containing the anionic moiety $X-N(O^-)-N=O$ as a function of time.

Samples of two NO-releasing compounds, specifically the secondary amine $\{\text{Et}_2\text{N}-\text{N}(\text{N}=\text{O})-\text{O}\}\text{Na}$, referred to as DEANO, and the polyamine $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}_2-(\text{CH}_2)_4-\text{N}\{\text{N}(\text{N}=\text{O})-\text{O}\}-(\text{CH}_2)_3-\text{NH}_2$, referred to as SPERNO, were obtained from Dr. Larry Keefer at the National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD. Small quantities of the two compounds were dissolved separately in phosphate buffered saline at pH 11, and the resulting solutions were thoroughly purged with nitrogen gas to remove dissolved oxygen. The concentrations of the solutions were adjusted to obtain final NO concentrations of about 100 μM in the reaction cell.

One mg of fusinite powder ($d \leq 10 \mu\text{m}$) was suspended in 10 ml of phosphate buffered saline at pH 7.4 in a glass cell fitted with a septum cap. The fusinite suspension was purged with nitrogen gas to remove dissolved oxygen until no further reduction in the EPR linewidth for the fusinite was observed (about 30 minutes). The cell containing the purged fusinite suspension was then placed on a loop-gas resonator surface coil connected to an EPR spectrometer operating at L-band (1-2 GHz) as described previously (Nilges et al., Phys. Med. 2: 195-201. 1989.; Bacic et al., Magnetic Resonance Medicine 10: 266-272. 1989.).

A 0.2 ml portion of each of the solutions containing the NO-releasing compounds was injected into separate fusinite suspensions. EPR spectra of the fusinite were collected at 10-second intervals for DEANO and two-minute intervals for SPERNO, utilizing a computer-controlled data acquisition system. During the collection of the EPR spectra, the temperature was monitored with a thermocouple and stabilized at around 35°C.

Figure 2 shows the measurement of the variation in NO concentration with time for DEANO. The excellent agreement between the data obtained with fusinite and EPR and the data obtained by optical absorbance suggests that

the fusinite/EPR method accurately follows the rate of production of NO. It appears that during the initial 100 seconds following injection of DEANO into the reaction cell, scavenging of residual adsorbed oxygen by the first 5 NO molecules produced in the reaction may be taking place.

In a separate experiment, fusinite powder ($d \leq 10 \mu\text{m}$) was suspended in 10 ml of phosphate buffered saline at pH 7.4 in a glass cell fitted with a septum cap. 10 Then, 0.2 ml of DEANO in deoxygenated, phosphate buffered saline at pH 11 was injected into the cell, and, while the system was maintained at 20°C, the EPR linewidth was monitored as a function of time. The EPR linewidth decreased due to the reaction of $\text{NO}\cdot$ with the dissolved 15 oxygen. The reaction is believed to be $4\text{NO}\cdot + 2\text{H}_2\text{O} + \text{O}_2 \rightarrow 4\text{HNO}_2$ (Schwartz et al. In: Trace Atmospheric Constituents. J. Wiley & Sons, NY. 1983. Chapter 1 at page 107.). Nitrites, e.g., NO_2^- dissociated from HNO_2 in aqueous media, are nonparamagnetic species. The 20 rate-determining step is, therefore, the production of NO· from the decomposition of DEANO. The rate constant for this step is believed to be $2 \times 10^{-3}/\text{sec}$.

Similar good agreement between the data obtained with fusinite and EPR and the data obtained by optical 25 absorbance for SPERNO is shown in Figure 3, which shows the kinetics of the formation of NO from the compound.

After the DEANO reaction had gone to completion, 2 ml of pure oxygen gas was injected into the reaction cell containing the fusinite suspension to observe the effect 30 of added oxygen on the ΔB_{pp} of the fusinite. Figure 4 shows the immediate narrowing of the EPR linewidth following oxygen administration. This demonstrates the reactivity of NO and O_2 to form diamagnetic products like NO_2 , and the sensitivity of fusinite and EPR to this 35 chemistry. This NO/ O_2 reaction could be used as a chemical calibration means to titrate NO, if necessary, in particular applications.

EXAMPLE 4

This example describes the use of fusinite and EPR to measure NO in cells.

Chinese hamster ovary (CHO) cells were allowed to 5 endocytose fusinite in culture. The cells were then placed in phosphate buffered saline at pH 7.4 in a glass cell fitted with a septum cap. An initial decrease in the EPR linewidth for fusinite was observed before the introduction of DEANO solution. The decrease in 10 linewidth is believed to reflect cellular respiration, i.e., the consumption of oxygen by the cells. The cells were allowed to respire until the linewidth was about 0.8 G, which indicates that almost all of the oxygen in the system had been consumed. Then 0.2 ml of DEANO 15 dissolved in deoxygenated, phosphate buffered saline at pH 11 was injected into the reaction cell containing the fusinite. The reaction cell containing the CHO cells was placed on a loop-gas resonator surface coil connected to an EPR spectrometer operating at L-band (1-2 GHz). The 20 temperature was maintained at 20°C in order to slow cellular metabolism. Figure 5 shows the initial decrease and subsequent increase in the EPR linewidth for fusinite over time as measured in the CHO cells. Following the introduction of DEANO, the kinetics of the reaction were 25 as follows: DEANO k_1 b(NO \cdot) k_2 C. C is assumed to be a nonparamagnetic species. In this equation, $k_1 = 2.5 \times 10^{-3}/\text{sec}$ and $k_2 = 6.7 \times 10^{-4}/\text{sec}$, based on the best fit of the curve to the data points and the condition that the maximum [NO \cdot] curve will occur when $t_{\max} = 30 \ln(k_2/k_1)/(k_2-k_1)$.

EXAMPLE 5

This example describes the use of fusinite and EPR to measure NO in an intact organism.

35 Fine particles of fusinite may be suspended in phosphate buffered saline at pH 7.4 and injected into a live mouse. The injection may be localized or systemic.

If systemic, the fusinite should be allowed to circulate throughout the body. A surface coil, operating at L-band, then may be placed in contact with a localized region of the body or the entire body. In connection 5 with an EPR spectrometer calibrated to measure the variation in the EPR linewidth for fusinite in the presence of NO, the location of NO production in the mouse may be determined by detecting variation in the EPR linewidth for the fusinite present in the body of the 10 mouse. Such a technique also may be used to quantify the NO produced and to image tumors, which are known to produce NO and which may be present in the body.

EXAMPLE 6

15 This example illustrates the effect on EPR linewidth for ¹⁵N-perdeutero TEMPONE as a function of NO concentration.

The EPR linewidth for ¹⁵N-perdeutero TEMPONE was observed as a function of NO concentration utilizing a 20 dynamic vacuum system that allowed a small quantity of ¹⁵N-perdeutero TEMPONE to be placed in an X-band (8.9-9.6 GHz) EPR cavity. Quantities of NO gas (ACS reagent grade, Matheson, Rutherford, NJ) were admitted to the system and the equilibrium gas pressure was measured 25 using a Varian Thermatron thermocouple gauge calibrated against a mercury McLeod gauge. EPR spectra were obtained for ¹⁵N-perdeutero TEMPONE at various equilibrium NO pressures using an EPR spectrometer (Varian, Palo Alto, CA). Figure 6 shows the variation in EPR linewidth 30 for ¹⁵N-perdeutero TEMPONE subjected to varying concentrations of NO.

EXAMPLE 7

35 This example describes an appropriate procedure for the use of TEMPO and EPR in the measurement of NO concentration in solution as a function of time.

An EPR system can be initially calibrated for TEMPO in the presence of NO in a manner similar to that described in Example 2 for fusinite in the presence of NO. A solution containing 1 mM TEMPO in phosphate buffered saline at pH 7.4 would be then placed in a glass cell fitted with a septum cap. The TEMPO solution would then be purged with nitrogen gas to remove dissolved oxygen until no further reduction in the EPR linewidth for the TEMPO was observed. The cell containing the purged TEMPO solution would be then placed on a loop-gas resonator surface coil connected to an EPR spectrometer operating at L-band.

A suitable portion, e.g., 0.2 ml, of a sample containing an unknown concentration of NO would be injected into separate TEMPO solutions. EPR spectra of the TEMPO would be collected at set intervals, utilizing a computer-controlled data acquisition system. During the collection of the EPR spectra, the temperature should be monitored with a thermocouple and stabilized at an appropriate temperature, e.g., about room temperature. The EPR linewidth for TEMPO would be expected to broaden in the presence of NO in the same manner as that of fusinite, thereby allowing for measurement of the NO concentration in the sample.

25

All of the publications identified herein are hereby incorporated by reference in their entireties.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred method may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of detecting nitric oxide, which method comprises contacting a sample of unknown nitric oxide concentration with a spin-labeled material, subjecting said unknown sample and spin-labeled material to EPR spectroscopy under hypoxic conditions to obtain an EPR signal having a linewidth, and comparing said EPR linewidth for said unknown sample and spin-labeled material to the EPR linewidth for said spin-labeled material in the presence of a sample of known nitric oxide concentration in order to detect the presence of nitric oxide.
- 15 2. The method of claim 1, wherein the spin-labeled material is selected from the group consisting of coal derivatives, burnt or partially combusted cellulose, irradiated gas-permeable plastics, 2,2,6,6-tetramethylpiperidine-N-oxyl, 20 4,4-dimethyloxazolidine-N-oxyl, 2,2,5,5-tetramethylpyrrolidine-N-oxyl, and derivatives thereof.
- 25 3. The method of claim 2, wherein the spin-labeled material is fusinite.
- 30 4. The method of claim 2, wherein said spin-labeled material is selected from the group consisting of 2,2,6,6-tetramethylpiperidine-N-oxyl, a spin-labeled molecule isolated from a coal derivative, burnt or partially combusted cellulose, or an irradiated gas-permeable plastic, and derivatives thereof.
- 35 5. The method of claim 4, wherein said spin-labeled material is selected from the group consisting of 2,2,6,6-tetramethylpiperidine-N-oxyl, a

spin-labeled molecule isolated from fusinite, and derivatives thereof.

6. The method of claim 5, wherein said
5 spin-labeled material is
2,2,6,6-tetramethylpiperidine-N-oxyl or a derivative
thereof.

7. The method of claim 1, wherein said sample of
10 known nitric oxide concentration is devoid of nitric
oxide and said EPR linewidth for said unknown sample and
spin-labeled material is compared to the EPR linewidth
for said spin-labeled material in the presence of said
sample devoid of nitric oxide in order to detect the
15 presence or absence of nitric oxide.

8. The method of claim 1, wherein said EPR
 linewidth for said unknown sample and spin-labeled
 material is compared to the EPR linewidth for said
20 spin-labeled material in the presence of at least one
 sample containing nitric oxide in a known concentration
 to determine the concentration of nitric oxide in said
 unknown sample.

25 9. The method of claim 8, wherein said EPR
 linewidth for said unknown sample and spin-labeled
 material is compared to the EPR linewidth for said
 spin-labeled material in the presence of more than one
30 sample containing nitric oxide in different known
 concentrations to determine the concentration of nitric
 oxide in said unknown sample.

10. The method of claim 1, wherein said sample is a
 biological sample.

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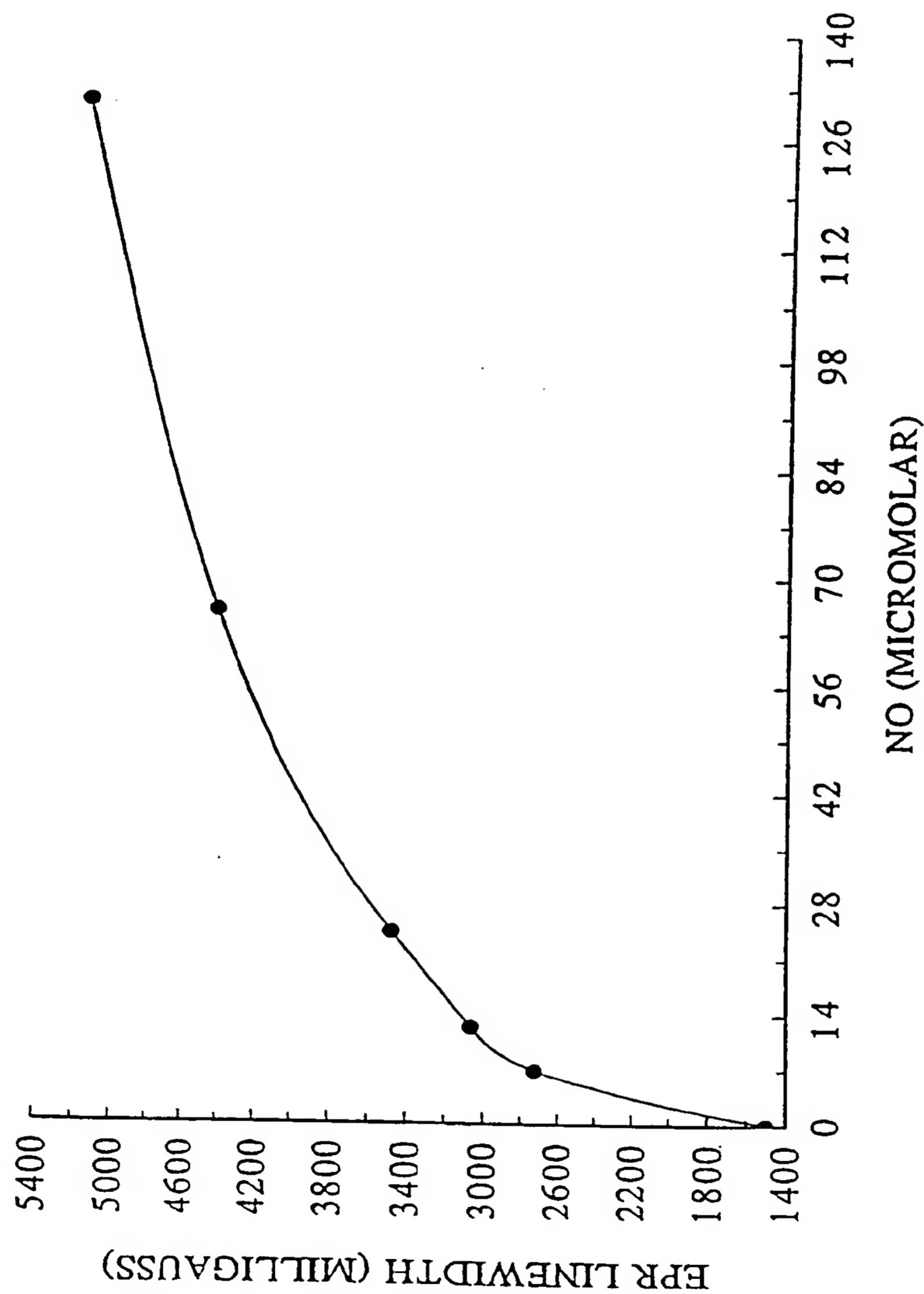


Fig. 1

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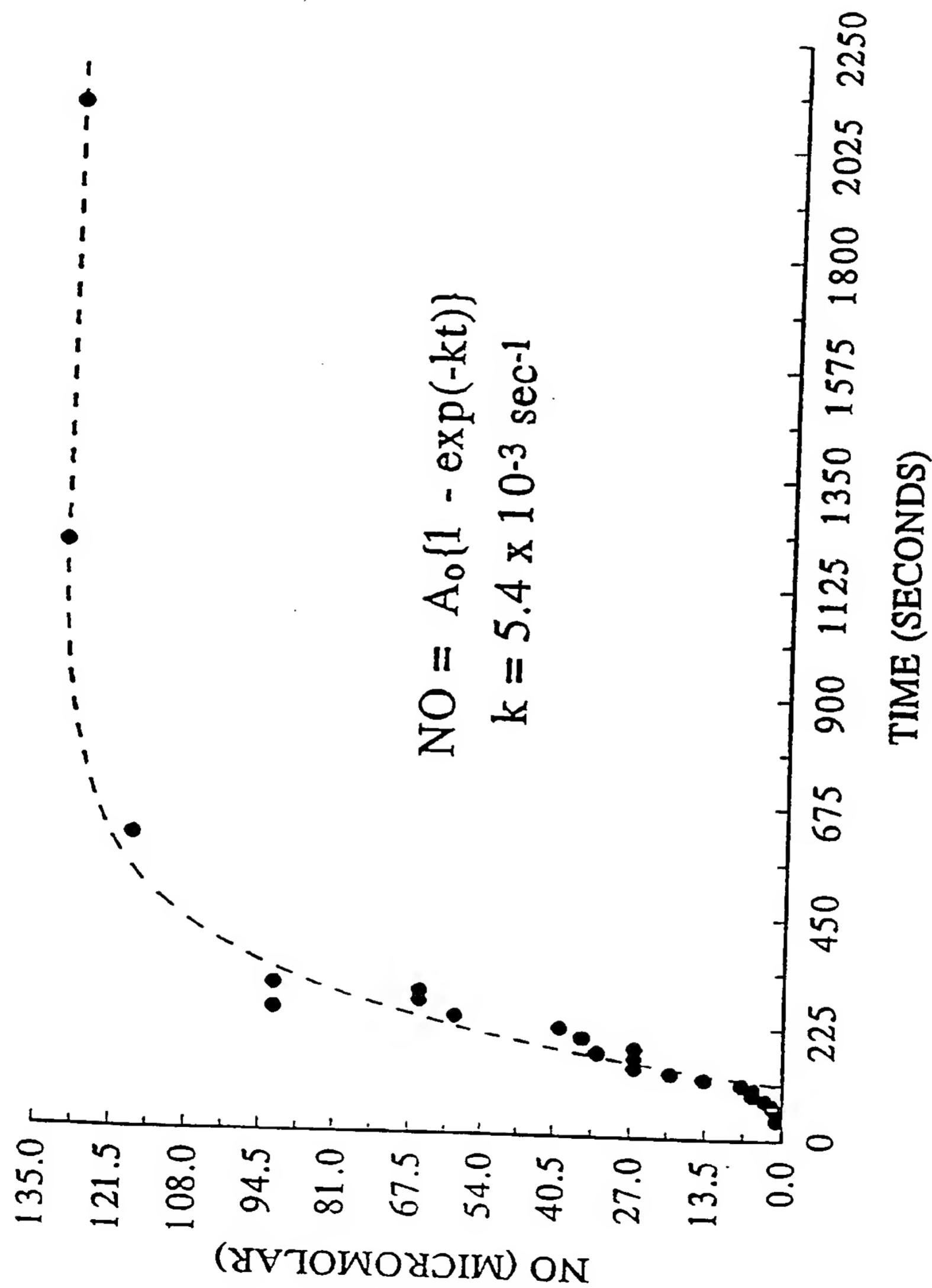


Fig. 2

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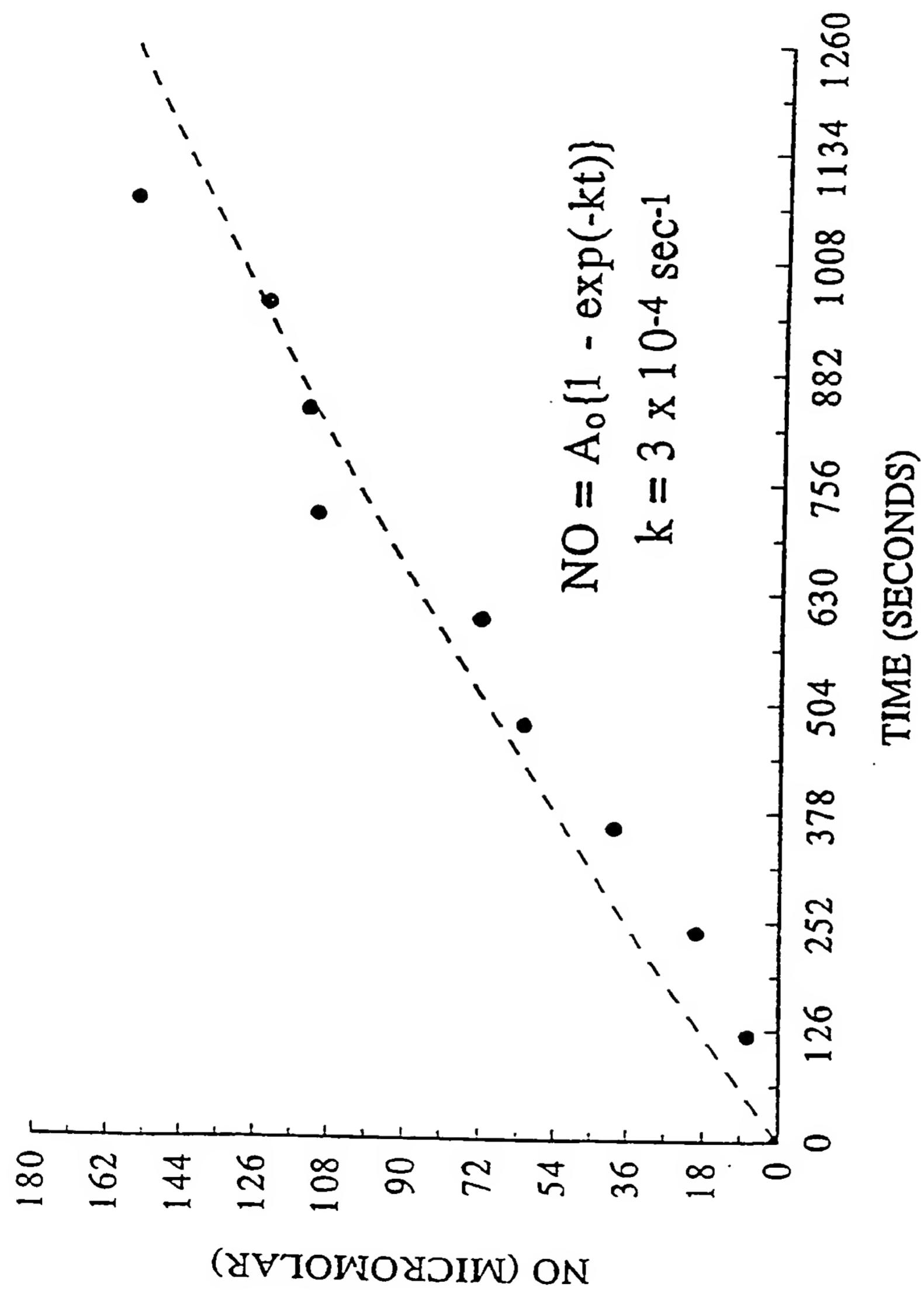


Fig. 3

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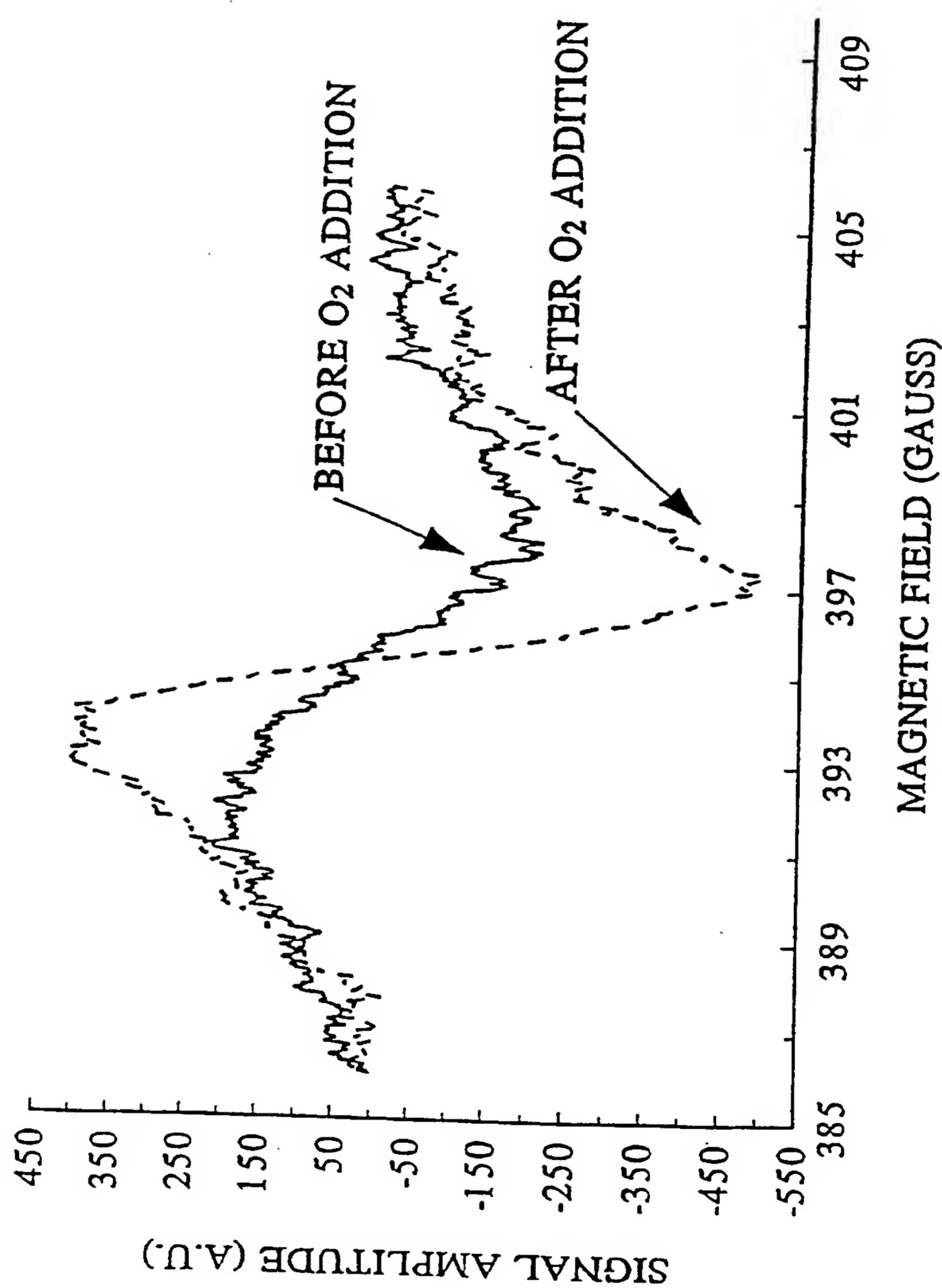


Fig. 4

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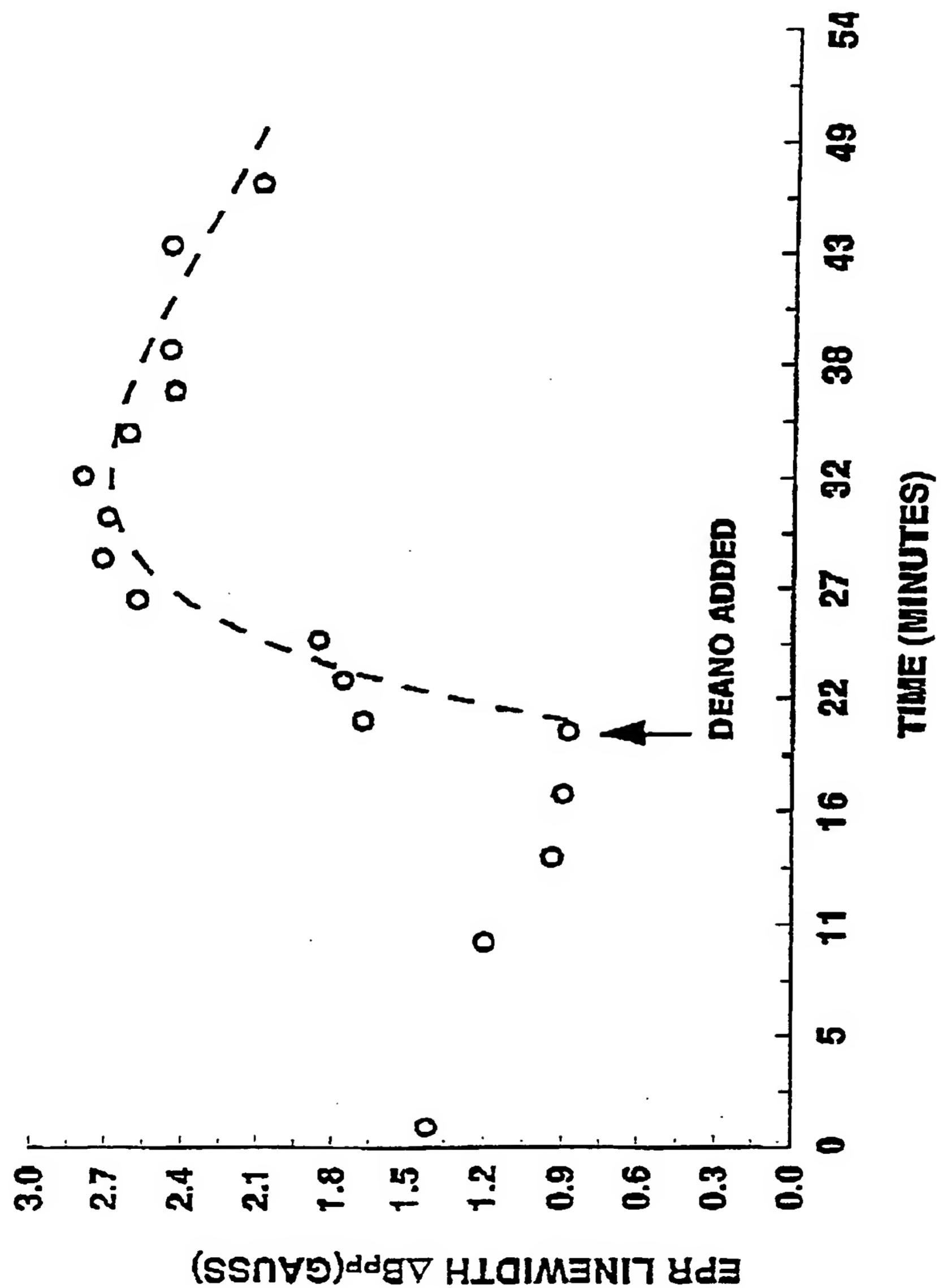


Fig. 5

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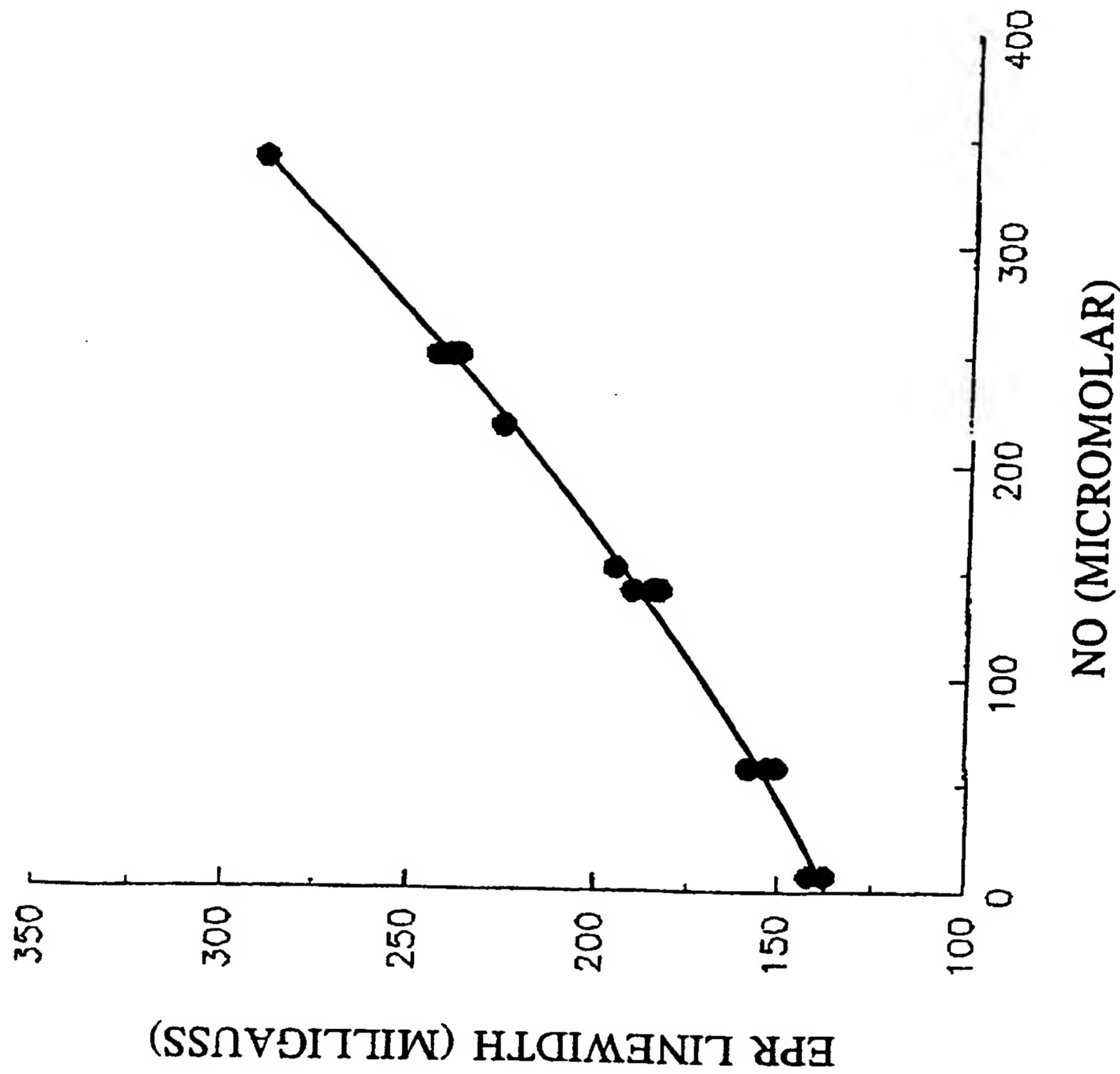


Fig. 6

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/06868

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 G01R33/60; A61K49/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	G01R ; A61K ; G01N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	<p>TETRAHEDRON LETTERS vol. 27, no. 39, 1986, OXFORD GB pages 4795 - 4798</p> <p>M. GYÖR ET AL. 'SPIN TRAPPING REACTIONS WITH NITRIC OXIDES. V. REACTIONS WITH UNSATURATED MACROMOLECULAR CHAINS- A NEW SPIN LABELING METHOD' see the whole document</p> <p>---</p> <p>PHYSICS IN MEDECINE AND BIOLOGY. vol. 34, no. 9, 1 September 1989, LONDON GB pages 1317 - 1323</p> <p>S. ISHIDA ET AL. 'IN VIVO ANALYSIS OF NITROXIDE RADICALS INJECTED INTO SMALL ANIMALS BY L-BAND ESR TECHNIQUE' see the whole document</p> <p>---</p>	1,2,10
A		1,2,4,10
		-/-
<p>¹⁰ Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed</p> <p>¹¹ T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family</p>		
IV. CERTIFICATION		
1 Date of the Actual Completion of the International Search 26 OCTOBER 1993		Date of Mailing of this International Search Report 03.11.93
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer HORAK G.I.

III. DOCUMENTS CONSIDERED TO BE RELEVANT		(CONTINUED FROM THE SECOND SHEET)
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF MAGNETIC RESONANCE. vol. 85, no. 1, 15 October 1989, ORLANDO, MN US pages 50 - 59 R.K. WOODS ET AL. 'SPECTRAL-SPATIAL ESR IMAGING AS A METHOD OF NONINVASIVE BIOLOGICAL OXIMETRY' see the whole document ---	1,2,10
A	MAGNETIC RESONANCE IN MEDICINE. vol. 20, no. 2, 1 August 1991, DULUTH, MN US pages 333 - 339 H.M. SWARTZ ET AL. 'MEASUREMENTS OF PERTINENT CONCENTRATIONS OF OXYGEN IN VIVO' cited in the application see the whole document ---	1-3,10
A	MAGNETIC RESONANCE IN MEDICINE. vol. 10, no. 2, 1 May 1989, DULUTH, MN US pages 266 - 272 G. BACIC ET AL. 'IN VIVO LOCALIZED ESR SPECTROSCOPY REFLECTING METABOLISM' cited in the application see the whole document ---	1,2,10
A	SCIENCE vol. 227, no. 4686, 1 February 1985, LANCASTER, PA US pages 517 - 518 L.J. BERLINER ET AL. 'MAGNETIC RESONANCE IMAGING OF BIOLOGICAL SPECIMENS BY ELECTRON PARAMAGNETIC RESONANCE OF NITROXIDE SPIN LABELS' see the whole document ---	1,2,4,10
A	US,A,4 593 248 (J.S. HYDE ET AL.) 3 June 1986 see column 2, line 3 - column 3, line 25 ---	1,2,10
A	US,A,4 099 918 (J.F.W. KEANA) 11 July 1978 see column 1, line 4 - column 2, line 60 -----	1,2,4,10

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9306868
SA 77142

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4593248	03-06-86	None	
US-A-4099918	11-07-78	None	

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